Trypanosome CNOT10 is essential for the integrity of the NOT deadenylase complex and for degradation of many mRNAs

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ABSTRACT

The degradation of most eukaryotic mRNAs is initiated by removal of the poly(A) tail, and the major deadenylase activity is associated with the CCR4/CAF1/NOT complex (NOT complex). We here study the role of CNOT10, a protein that is found in human and trypanosome, but not in yeast, NOT complexes. Trypanosome (Tb) CNOT10 is essential for growth. TbCNOT10 interacted with the deadenylase TbCAF1 and the scaffold protein TbNOT1; TbCAF1 also interacted with TbNOT1 in a yeast two-hybrid assay. In both trypanosomes and human embryonic kidney cells, approximately half of CAF1 was associated with the NOT complex. Depletion of CNOT10 from human cells did not affect this association. In contrast, depletion of TbCNOT10 in trypanosomes caused a decrease in the level of TbNOT1, detachment of TbCAF1 from the complex and pronounced stabilization of most trypanosome mRNAs. Artificial tethering of TbCAF1 to a reporter mRNA in vivo resulted in mRNA degradation, and this was not affected by TbCNOT10 depletion. We conclude that in trypanosomes, TbCNOT10 may stabilize the interaction between TbCAF1 and the NOT complex. The results further suggest that TbCAF1 is only able to deadenylate mRNA in vivo if it is recruited to the mRNA through other NOT complex components.

INTRODUCTION

The degradation of mRNAs is important for control of gene expression. In eukaryotes, the degradation of most mRNAs is initiated by removal of the poly(A) tail. Decay then proceeds either from the 3' end, by the exosome, or from the 5' end by decapping and subsequent digestion by the exoribonuclease XRN1 (1). Three cytosolic deadenylases have been described in eukaryotes: the poly(A) ribonucleases; the poly(A) nuclease Pan2 with its co-factor Pan3; and CCR4 and CAF1, both of which are part of the CCR4–CAF1–NOT complex (hereafter simply called ‘the NOT complex’) (2). The NOT complex has been found to be the most important effector of mRNA deadenylation (3–5). In addition, it has been implicated in the regulation of transcription, the DNA damage response, nuclear surveillance, mRNA export and translation repression (6).

In all eukaryotes examined so far, the NOT complex consists of at least seven subunits. In yeast, the universally conserved subunits are called Caf1p, Caf40p and Not1p, Not2p and Not5p (5,7–11). Not1p (human CNOT1, Drosophila and trypanosome NOT1) acts as a platform for the remaining core subunits. In human cells, it is also recruited by GW182/TNRC6 for miRNA-induced mRNA decay (12) and by Tis11 proteins, which mediate degradation of mRNAs with AU-rich elements (6,13). The central role of NOT1 as a scaffold is shown by the fact that in human cells and yeast, its depletion disrupts the complex and abrogates mRNA deadenylation (14,15). The major deadenylase activity in the human, Trypanosoma and Drosophila complexes is CAF1; there are two paralogues in humans, called Caf1a/b or CNOT7 and CNOT8. Animal cells and yeast have an additional deadenylase called CCR4 or Ccr4p; in yeast, Ccr4p is more active than Caf1p (4–6). Recent crystal structures for relevant fragments of the yeast and human Not1 and Caf1 proteins have shown that they are held together mainly through hydrophobic interactions (16,17).

The functions of the remaining core subunits are not clear. Yeast has two proteins equivalent to NOT5: Not3p and Not5p. Although these are 44% identical, the corresponding mutant strains showed specific gene expression changes, suggesting that the two subunits have distinct roles (15). NOT2 (human CNOT2) is important for complex integrity, as it seems to link NOT3/5 to NOT1 protein interactions.

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The role of Caf40p (human Rcd-1) is unknown, although Rcd-1 can dimerize and bind single-stranded DNA (20). In yeast, Not4p is associated with the NOT complex (21); it is an E3 ligase involved in degrading aberrant polypeptides that lack a stop codon (22) and may act independently of the other subunits. The homologues in human cells (CNOT4) and Drosophila are not stably associated with the complex (9,10) and there is no homologue in trypanosomes (5).

The remaining subunits are less widely distributed in evolution. Caf130p, which is found exclusively in fungi, interacts with the N- and C-termini of Not1p, but is not important for the stability of the complex (15,23). Two more subunits have been identified in human cells: TAB182 and C2ORF29 (9). This article concentrates mainly on CNOT10, which has been described in human cells but is absent from fungi. Tandem affinity purification (TAP) of human CNOT10 retrieved all core subunits of the complex, but not the deadenylases (9); apart from that nothing is known about CNOT10 function.

The parasite Trypanosoma brucei, and related kinetoplastid protists, have an exceptional genome organization. Protein-coding genes are arranged in large polycistronic units transcribed by RNA polymerase II (24). Cleavage and maturation of the precursor mRNA are initiated by trans-splicing, which places identical leader sequences at the 5'-end of every mRNA; this is coupled to polyadenylation of the mRNA immediately upstream (25,26). As a consequence of this gene arrangement, the parasite lacks transcriptional control of individual protein-coding genes. Transcript levels are determined mainly by gene copy number and mRNA degradation rates (27). We have previously shown that the degradation of most trypanosome mRNAs is initiated by deadenylation (27). TAP of T. brucei CAF1 identified all core subunits of the complex, including a distant relative of Homo sapiens (Hs) CNOT10 (5). In this report, we analyse the role of TbCNOT10 in trypanosomes and compare some results with those seen for its counterpart in human cells.

MATERIALS AND METHODS

Cell culture

The experiments were done with bloodstream-form T. brucei that stably express the tetracycline repressor, with or without T7 polymerase expression. Culturing, transfections and RNAi experiments were conducted as described earlier (28). mRNA decay was analysed after 24 h of RNAi targeting TbCNOT10. Cells were treated with Sinefungin (final concentration 2 μg/ml) for 5 min, and then actinomycin D was added to a final concentration of 10 μg/ml.

Human embryonic kidney (HEK) 293 cells were cultured in Dulbecco's-modified Eagle medium (DMEM) containing 10% fetal bovine serum (PAA Laboratories), 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin (all PAN Biotech) at 37°C/5% CO2. Cells were transfected with DNA using polyethyleneimine (PEI) (Polysciences Europe; 1 mg/ml, pH 7.0) at a ratio of 1:2 (DNA:PEI) in serum-free DMEM without antibiotics. For transfection of siRNAs, Lipofectamine RNAiMax (Invitrogen) and Optimem (Gibco) were used according to the manufacturer's protocol. siRNAs were transfected at a concentration of 100 nM with Lipofectamine RNAiMax twice over a time period of 4 days. Medium was changed to regular DMEM 4 h after transfection of DNA.

Lists of plasmids, primers and siRNAs used in this article are in Supplementary Table S1.

RNA extraction and northern blot analysis

Total RNA was extracted using peqGold Trifast (peqLab, Germany). RNA was run on 1.5% formaldehyde gels or 4.5% polyacrylamide-urea gels and blotted onto Nytran membranes (GE Healthcare). RNase H digestion with oligo d(T) was adapted from the method described previously (5). Ten micrograms of RNA was mixed with 170 pmol oligo d(T) and RNase H buffer (New England Biolabs) and filled up with water to 50 μl. The mixture was denatured at 85°C for 5 min, incubated at 42°C for 10 min, then further cooled over 15 min at 32°C. One microlitre of RNasin® RNase Inhibitor (Promega) and 5 U RNaseH (New England Biolabs) were added and the reaction incubated at 37°C for 1 h.

Northern blots were hybridized with radioactively labelled DNA (Prime-IT RmT Random Primer Labelling Kit, Stratagene) or RNA (MAXIscript, Ambion) probes. Signals were measured using a phosphorimager. For trypanosome RNA, either the signal of the signal recognition particle (7SL) or a large subunit rRNA (LSU1) was used for normalization. The half-lives were estimated using only those segments of the time course that gave exponential decay curves (fitted with a linear correlation coefficient generally exceeding 0.9).

Co-immunoprecipitation

To make trypanosome extracts, 5 × 107 trypanosomes were re-suspended in immunoprecipitation (IP) lysis buffer (10 mM NaCl, 10 mM Tris–HCl, pH 7.5, 0.3% IGEPAL and protease inhibitors [Protease Inhibitor Mixture [ethylenediaminetetraacetic acid (EDTA)-free]; Roche Applied Science]) and passed five times through a 27-gauge syringe. The resulting extracts were centrifuged for 20 min at 4°C, at 16000 × g. The supernatant was transferred into a new tube and the salt concentration was adjusted to 180 mM NaCl. To test the RNA dependence of interactions, RNaseA was added to the lysis and wash buffers at a concentration of 200 μg/ml, with or without RNasin® Ribonuclease inhibitor (Promega) at a concentration of 48 μg/ml.

For IP, 30 μl of anti-V5-antibody- or anti-c-myc-antibody-coupled agarose (both from Bethyl Laboratories) was washed four times with 1× phosphate buffered saline (PBS) and once with IP lysis buffer adjusted to 180 mM NaCl at 4°C, with centrifugation at 0.9 × g for 2 min. A sample equivalent to 5 × 106 cells was taken from the input lysate and 2× Laemmli buffer was added. The remainder of the lysate was then added to the beads, and the mixture incubated for 1–1.5 h at 4°C with...
rotation. After centrifugation, the supernatant was transferred to another tube and a sample equivalent to 5 × 10^7 cells was again taken and 2× Laemmlı buffer was added. The remaining beads were washed four times for 5 min at 4°C with salt-adjusted lysis buffer, with intermediate centrifugation, then boiled in 2× Laemmlı buffer.

For human cells, 24 h after transient transfection, HEK293 cells from a confluent 10 cm dish were collected and lysed in 400 μl ice-cold RNA-IP lysis buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 1 mM MgCl₂, 2% NP-40 and 10% glycerol with freshly added protease inhibitors [Complete; Roche]). Nuclei were removed by centrifugation at 500 × g for 5 min at 4°C. A total of 30 μl of streptavidin sepharose beads (GE Healthcare) was added for an additional 1.5–2 h and washed six times in NET2 buffer (50 mM Tris [pH 7.5], 150 mM NaCl and 0.5% Triton X-100). Protein complexes were eluted with 50 μl sodium dodecyl sulphate (SDS) sample buffer with 100 mM dithiothreitol (DTT). Proteins were resolved on 5–20% gradient polyacrylamide gels and transferred onto a 0.2-μm pore size nitrocellulose membrane (Pezlab) for western blotting. Horseradish peroxidase-coupled secondary antibodies (Jackson Immunoresearch) in combination with Western Lightning-enhanced chemiluminescence substrate (Perkin Elmer) were used for detection. Streptavidin sepharose beads (GE Healthcare) were used to purify myc-Strep-tagged (myc-SG) proteins.

Two-hybrid analysis
Complete trypanosome open reading frames (ORFs) were cloned into pGADT7 (GAL4 activation domain vector) and pGBK7 (GAL4 DNA-binding domain vector) (Matchmaker 3 System, Clontech). The two-hybrid yeast strain AH109 was then transformed with these plasmids in all possible combinations. The expression of the fusion protein was confirmed by western blot analysis using antibody to the HA epitope of the GAL4 activation domain fusion proteins, and antibody to the c-myc epitope of the GAL4 DNA-binding domain fusions. Transformants were selected on four drop-out (SD/-Trp/-Leu/-His/-Ade) culture plates after 4 days of incubation at 30°C, and the resulting positive clones were assayed for β-galactosidase activity using a colony-lift filter assay. Alternatively, reporter activation was tested by replica plating on triple drop-out plates: SD/-Trp/-Leu/-His (medium stringency). As negative controls for self-activation, we used combinations of the TbNOT-complex subunits with CLONTECH vectors pGAD-T-antigen and pGBK7-p53. Further, p53 (pGBK7-p53) and SV40 large T-antigen (pGADT7-T) cotransformants were used as positive controls, whereas lamin C (pGBK7-Lamin) and pGADT7-T cotransformants were used as negative controls.

Glycerol gradients
Trypanosomes (3 × 10^7), or HEK293 cells from a confluent 10 cm dish, were washed with 1× PBS, then frozen in liquid nitrogen and stored at −80°C until use. The cell pellets were thawed on ice and re-suspended in ice-cold buffer containing 10 mM Tris–HCl, pH 7.6, 10 mM NaCl, 10 mM MgCl₂ and protease inhibitors (Protease Inhibitor Mixture [EDTA-free]; Roche Applied Science), then forced five times through a 27-g needle. IGEPAL CA-630 (Sigma) was added to a final concentration of 0.1% and the cells were again forced through the needle five times. The lysate was centrifuged first for 20 min at 4°C, 10 000 × g, then the supernatant was spun for 1 h at 100 000 × g at 4°C. The final supernatant was layered onto a 12-ml 10–30% glycerol gradient (200 mM KAc, 20 mM Tris–HCl, pH 8.0, 5 mM MgAc, 1 mM DTT and protease inhibitors [Protease Inhibitor Mixture [EDTA-free]; Roche Applied Science]), which was then spun at 229 900 × g for 17 h at 4°C. Twenty-seven fractions were taken from the top at 23-s intervals. The fractions were incubated on ice overnight with 15% trichloroacetic acid, 0.1% DOC and 10 μg bovine serum albumin (BSA). Precipitates were washed with acetone and re-suspended in Laemmlı buffer.

Tandem affinity purification
Trypanosomes 2.5 × 10^10 were harvested and used for TAP as previously described (29). The eluate was run 1 cm into a 10% SDS–polyacrylamide gel electrophoresis (PAGE) resolving gel and stained with colloidal Coomassie. The protein-containing gel area was cut into five slices, which were analysed by mass spectrometry.

Protein detection
Trypanosomes or HEK cells were washed once with PBS then dissolved in 2× Laemmlı buffer. Protein samples were run on a SDS-PAGE, then blotted onto an Optitran BA-S 85 reinforced NC 0.45μm membrane (Whatman, Dassl, Germany). The blots were probed with appropriate antibodies and then developed with Western Lightning®-ECL solution (Perkin Elmer).

Immunofluorescence (IF) microscopy was conducted as described earlier in (5). The following antibodies were used in western blotting: monoclonal mouse Anti-V5 (Santa Cruz), monoclonal mouse anti-e-myc (Santa Cruz), monoclonal mouse anti-GFP (Santa Cruz), ECL™ Anti-Rabbit IgG (GE Healthcare), ECL™ Anti-Mouse IgG (GE Healthcare), polyclonal rabbit antibodies anti-myc (A-14, sc-789, Santa Cruz), anti-HsCNOT10 (15938-1-AP, Protein Tech) and anti-HsCNOT1 (14276-1-AP, Protein Tech); polyclonal anti-trypanosome aldolase (30). Polyclonal rabbit anti-Caf1a was kindly provided by Ann-Bin Shyu (University of Texas, Houston, TX, USA) and monoclonal antibody to trypanosome tubulin was from Keith Gull (31).

We expressed recombinant His-tagged TbCNOT10 (full length) in Escherichia coli but it was very poorly soluble and we were unable to elute it specifically from the nickel column. A preparation of adequate quality for immunization was not obtained.

Database searches
To find potential homologues of Tb927.10.8720, (old number Tb10.6k15.1820), the protein sequence was analysed by BLASTp, psi-BLAST and tBLAST (32). We
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**RESULTS**

**Phylogenetic sequence analysis of TbCNOT10**

The protein encoded by trypanosome locus *Tb927.10.8720* was previously identified as a potential component of the NOT complex through TAP of *TbCAF1* (5). It is 555 amino acids long, with tetratricopeptide repeat (TPR)-like domains in positions 199–313 and 471–550. BLASTp and psi-BLAST searches on the whole Genbank database (excluding kinetoplastids) identified, as matches, >40 orthologs of CNOT10 in plants and animals, with *E*-values ranging from 3 × 10⁻⁴ to 1 × 10⁻⁷. The regions that matched covered 20–60% of the proteins, with identities of 20–30%. Reciprocal BLASTp of human CNOT10 against the *T. brucei* genome yielded *Tb927.10.8720* as the best match (*E*-value 3 × 10⁻⁴). Most matches with other CNOT10s were located in the beginning of the first *TbCNOT10* TPR domain (Figure 1A). We also did directed BLASTp searches using human or trypanosome CNOT10 to probe different eukaryotic phyla and kingdoms: we were unable to find equivalents to CNOT10 in Apicomplexa, Diplomonadida, Alveolates and fungi, but it is present in plants. We concluded that *Tb927.10.8720* might be homologue of human CNOT10, so we provisionally named it *TbCNOT10*.

**TbCNOT10 is a component of the NOT complex**

To confirm *TbCNOT10* as a member of the NOT complex, we conducted IPs. We used trypanosomes expressing tagged versions. The *TbCNOT10* gene was tagged *in situ*, by homologous recombination with a sequence encoding an N-terminal V5-tag. *TbCAF1* with a C-terminal myc tag (CAF1-myc) was expressed from a tetracycline-inducible promoter. A pull-down of V5-tagged *TbCNOT10* (V5-*TbCNOT10*) resulted in coprecipitation of *TbCAF1*-myc (Figure 1B) and vice versa (Supplementary Figure S1). RNaseA treatment did not disrupt the interaction (not shown).

In order to identify further proteins that are stably associated with *TbCNOT10*, we generated trypanosomes in which a TAP-tag-coding sequence was integrated directly upstream of the *TbCNOT10* ORF. In IF microscopy, TAP-tagged *TbCNOT10* was detected in a rather granular pattern in the cytoplasm (Figure 1D). After TAP of *TbCNOT10*, all associated proteins were analysed by mass spectrometry (Supplementary Table S2). We found all previously known members of the trypanosome NOT complex except *TbCAF1*; they included the uncharacterized *Tb927.8.1960* protein, which had been seen in the previously published purification (5). These results confirmed that *TbCNOT10* is a stable component of the trypanosome NOT complex. Previous results from human cells were similar: after purification of the TAP-tagged *HsCNOT10*, neither *HsCNOT6/6L* (CCR4) nor *HsCNOT7* (CAF1a) was found (9). *TbCAF40* (*Tb927.4.410*), which had not been found in our published purification using TAP-tagged CAF1 (5), was detected in the CNOT10 preparation. We also found *TbDHH1* (as seen previously with *TbCAF1*).

There was also a number of novel potential NOT-associated proteins. These included three ATP-dependent DEAD/H RNA helicases (*Tb927.3.2600*, *Tb927.8.1510* and *Tb09.211.3510*), two of which are potential homologues of yeast Ded1p and Dbp2p (34). We have, however, found the putative Ded1p homologue (*Tb09.211.3510*) in several other purifications, so the specificity of the interaction is unclear. PUF2 was pulled down, but subsequent co-IPs failed to show a specific interaction (not shown). The only other interaction of note was with a potential homologue of the yeast karyopherins Pse1p and Kap123p, encoded by *Tb11.01.7010*; this may have parallels in human cells since some components of the human NOT complex interact with the nuclear pore (9). Additional possible interaction partners included kinetoplastid-specific proteins of unknown function (see Supplementary Table S2).

**Most NOT complex components are essential for the survival of *T. brucei***

To see whether *TbCNOT10* was essential in trypanosomes, we made permanent cell lines with tetracycline-inducible RNAi targeting *TbCNOT10* mRNA. Using the parasite form that lives in the mammal (bloodstream forms), we observed a severe proliferation defect within 36 h after RNAi induction (Figure 1C). In the slower growing procyclic form, a parasite stage that multiplies in Tsetse flies, it took ~60 h to observe a decrease in cell numbers. For comparison, we knocked down *TbNOT2* (*Tb927.6.850*, *TbNOT3*/5 (*Tb927.3.1920*) and *TbCAF40* (*Tb927.4.410*) RNAs. Proliferation defects were seen for *TbNOT3*/5 and *TbCAF40*, but not for *TbNOT2* (Supplementary Figure S2). In contrast, in the published high-throughput RNAi screen, *TbNOT2* RNAi also gave a growth defect (35). Our negative result for *TbNOT2* could have been due to inadequate RNAi. The high-throughput screen (35) revealed growth defects for *Tb927.8.1960* only during differentiation, and we too saw no defect after bloodstream-form RNAi (not shown).

Knowing that CNOT10 was essential, we could confirm the functionality of TAP-tagged CNOT10. In the line expressing the tagged version, we knocked out the remaining wild-type gene and found that the cells grew normally.

**Depletion of *TbCNOT10* inhibits mRNA turnover and deadenylation**

We next investigated the effect of *TbCNOT10* depletion on mRNA degradation, by inhibiting mRNA synthesis and processing, then measuring mRNA levels by northern blotting. Since all trypanosome mRNAs have the 39mer-spliced leader at the 5'-end, the turnover of bulk mRNA can be assessed by hybridization of northern blots with a spliced leader probe (36). Using this method with wild-type trypanosomes, half of the mRNA remained...
after 30 min. After depletion of CNOT10, however, virtually no mRNA degradation was seen (Figure 2A). This indicated that TbCNOT10 was important for overall mRNA degradation. Intriguingly, the spliced leader precursor (arrow) also appeared to have been stabilized.

Next, we studied three individual mRNAs, encoding actin (ACT), EP procyclin (EP) and histone H4 (HISH4). Degradation of each was inhibited (Figure 2B–D). In TbCNOT10-depleted trypanosomes, both degradation of ACT mRNA was delayed. Moreover, the steady-state level of ACT increased ~6-fold after TbCNOT10 depletion (Figure 2B). These effects were even more marked for EP mRNA, with the steady-state level increasing 3–4-fold (Figure 2C). We previously showed that EP mRNA degradation is biphasic, with an initial rapid degradation mediated by the 5′–3′ pathway, followed by a deadenylation-dependent phase which was inhibited by CAF1 RNAi (37). Exactly the same was seen now for CNOT10 RNAi (Figure 2C). The HISH4 mRNA was also more stable after the RNAi, but there was no evident change in the steady-state level (Figure 2D, lanes 2 and 3). To visualize deadenylation, we ran a sample on a polyacrylamide gel, flanked by marker lanes of fully deadenylated mRNA (treated with oligo d(T) and RNase H). Several polyadenylation sites are used (Figure 2D, lanes 1 and 10), and full-length polyadenylated mRNA forms a smear between 500 and 700 nt (lanes 2 and 3) (5). After transcription inhibition, the smear becomes progressively shorter (lanes 6 and 8), and this process was clearly delayed in cells with CNOT10 RNAi (lanes 7 and 9). These results indicate that TbCNOT10 is important for deadenylation and degradation of mRNAs in T. brucei.

**TbCNOT10 is important for association of TbCAF1 with the NOT complex**

To assess the effect of TbCNOT10 depletion on the NOT complex, we analysed migration of different complex components by glycerol gradient centrifugation. For these experiments, we used equal mixtures of three cell lysates. Two lysates were always from cells expressing *in situ* V5-tagged versions of TbNOT1 or TbCNOT10. These were used to follow the position of the complex. Both TbNOT1 and TbCNOT10 migrated between fractions 9 and 20 (Figure 3A). The third lysate came from trypanosomes with *in situ* V5-tagged CAF1, with or without...
"CNOT10 RNAi. In cells without RNAi, about half of the CAF1 was present in fractions 9–20 of the gradients, while the remainder was in lighter fractions (Figure 3A, C and D). We concluded that the NOT complex was concentrated in fractions 13–18, but that half of the CAF1 was not associated with it. In contrast, when we knocked down TbCNOT10 by RNAi, TbCAF1 no longer co-migrated with the complex at all (Figure 3B–D). This result suggested that TbCNOT10 is essential for the interaction of TbCAF1 with the rest of the NOT complex.

TbCNOT10 depletion reduces the level of TbNOT1
To define the interactions within the trypanosome NOT complex in more detail, we analysed them using the yeast two-hybrid system. The experiments showed that TbCNOT10 interacted with TbCAF1, TbNOT5 and the N- and C-terminal parts of TbNOT1 (Figure 4 and Supplementary Figure S3). The interaction between TbCNOT10 and TbNOT5 was seen only with TbCNOT10 as the prey and TbNOT5 as the bait. TbCAF1 interacted with TbCNOT10 and the N-terminal part of TbNOT1, but not with TbNOT5. Interaction of TbCAF1 with the C-terminal part of TbNOT1 was only seen in one direction, under low stringency conditions (Supplementary Figure S3).

To find out whether TbCNOT10 depletion directly affects the level of TbCAF1 or TbNOT1 in trypanosomes, we knocked down TbCNOT10 in cells expressing in situ-tagged V5-TbCAF1 or V5-TbNOT1, and measured the proteins by western blot. The amount of V5-TbCAF1 was not influenced by depletion of TbCNOT10 (Figure 5A, lanes 3 and 4), but TbNOT1 was decreased (Figure 5B, lanes 3 and 4).

The effect of TbCNOT10 depletion on TbCAF1 association was next investigated by co-IP. We first used trypanosomes with V5-tagged TbNOT1 and inducible TbCAF1-myc; note that the V5-NOT1 is likely to be at

Figure 2. Effect of TbCNOT10 depletion on mRNA degradation in trypanosomes. (A) Bulk mRNA turnover of wild-type cells and TbCNOT10-depleted cells was analysed by hybridization of northern blots with a spliced leader probe. Transcription was inhibited and the signal was measured for two time points (T = 0 and 30min). In each case, the signal of rRNA was used as a loading control. The northern blots from T = 0 and 30min originate from the same gel, but were cut due to the loading of other samples in between. (B–D) To find out whether TbCNOT10 depletion directly affecting the level of TbCAF1 and TbNOT1 in trypanosomes, we knocked down TbCNOT10 in cells expressing in situ-tagged V5-TbCAF1 or V5-TbNOT1, and measured the proteins by western blot. The amount of V5-TbCAF1 was not influenced by depletion of TbCNOT10 (Figure 5A, lanes 3 and 4), but TbNOT1 was decreased (Figure 5B, lanes 3 and 4).

The effect of TbCNOT10 depletion on TbCAF1 association was next investigated by co-IP. We first used trypanosomes with V5-tagged TbNOT1 and inducible TbCAF1-myc; note that the V5-NOT1 is likely to be at
Figure 3. Depletion of TbCNOT10 results in loss of TbCAF1 from the trypanosome complex. (A) Extracts of three different trypanosome lines (in situ V5-tagged TbCAF1, TbCNOT10 and TbNOT1) were mixed and run on a 10–30% glycerol gradient. The gradient was fractionated into 26 fractions. The additional bands between TbNOT1 and TbCNOT10 were degradation products of TbNOT1. Size indications are shown in figure (B) at the bottom. (B) Glycerol gradient of TbCNOT10-depleted cells with in situ V5-tagged TbCAF1. (C, D) Quantification of the signals from three independent gradients. (C) For each fraction, the level of signal is expressed as the percentage of the total signal on the blot. V5-TbCAF1 in TbCNOT10-depleted cells (dashed line; n = 3) and control (black line; n = 3). A comparison of the V5-TbCAF1 western blots signal from fractions 10–20 is shown in (D).

Figure 4. Yeast two-hybrid interactions in the trypanosome NOT complex. Left: minus and plus signs indicate the absence or presence of interaction detected by the activation of the His3 and LacZ reporters; multiple pluses indicate the activation of the Ade2, His3 and LacZ reporters. Lamin and T antigen (T) are negative controls. Right: illustration of the trypanosome NOT complex. Lines indicate interaction between different subunits, when the line is dashed, the interaction was shown only in one direction.
roughly endogenous levels whereas the CAF1-myc is over-expressed. Pull-down of TbCAF1-myc yielded V5-TbNOT1 as expected (Figure 6A, lane 6), though most of the V5-TbNOT1 appeared not to be associated with TbCAF1 (Figure 6A, lane 6). If all TbNOT1 is associated with TbCAF1, this could mean that there is much more untagged TbCAF1 than TbCAF1-myc in the cells; alternatively, perhaps some TbNOT1 is not associated with TbCAF1. When TbNOT10-depleted trypanosomes were used, the amount of co-precipitated TbNOT1 was reduced as expected (Figure 6A, lane 9). Thus, under the conditions used, TbNOT10 appeared to be limiting for the TbCAF1–TbNOT1 association. Using the same extracts, the reciprocal pull-down of V5-TbNOT1 yielded TbCAF1-myc as expected (Figure 6B, lane 6), although again most TbCAF1-myc remained in the supernatant (Figure 6B, lane 8), consistently with the glycerol gradient result and the over-expression of TbCAF1-myc. Puzzlingly, when TbNOT10-depleted trypanosomes were used for the V5-TbNOT1 pull-down, the amount of co-precipitated TbCAF1-myc was only slightly affected even though the amount of precipitated V5-TbNOT1 was lower (Figure 6B, compare lanes 6 and 9). This is difficult to explain because it is inconsistent with the other results; perhaps, it is connected with over-expression of TbCAF1-myc.

Because of this result, we also used the association of TbCAF40 to monitor complex integrity. In cells with normal amounts of TbCNOT10, precipitation of TbCAF1-myc resulted in co-precipitation of V5-TbCAF40 (Figure 6C, lane 6), but this no longer occurred in TbCNOT10-depleted trypanosomes (Figure 6A, lane 9). The results were confirmed by reciprocal pull down (Figure 6D, lanes 6 and 9).

Together, these results show that both TbCAF1 and TbCNOT10 interact directly with TbNOT1, and that the presence of TbCNOT10 enhances the interaction between TbCAF1 and TbNOT1. Depletion of TbCNOT10, with the accompanying reduction in the TbCAF1–TbNOT1 interaction, reduces the cellular level of TbNOT1.

**CAF1 deadenylation activity is not complex dependent**

One possible interpretation of all observations so far was that when TbCAF1 is not in the NOT complex, it is unable to initiate mRNA degradation. However, we had previously shown that recombinant TbCAF1 is active in deadenylation in vitro (5). There are two possible explanations for this discrepancy. Either TbCAF1 enzyme activity in vivo is dependent on, or stimulated by, interactions in the complex, or TbCAF1 relies on the complex for attachment to substrates. To distinguish between these possibilities, we created a cell line containing a constitutively expressed mRNA reporter, which consisted of a GFP ORF followed by six boxB elements and an actin 3′-UTR (GFP-B-ACT). In addition, the cell line inductively expressed a TbCAF1 fusion protein with a λN peptide at the N-terminus and myc at the C-terminus (λN-TbCAF1-myc). This hybrid protein should be ‘tethered’ to the GFP-B-ACT reporter RNA through the interaction between the B-boxes and the λN peptide. Upon induction of λN-TbCAF1-myc expression, GFP-B-ACT mRNA was destroyed (Figure 7, lanes 1, 2 and 7, 8). A control RNA with no boxB (GFP-ACT) was unaffected by λN-CAF1-myc expression (not shown).

So far the tethering result did not tell us whether TbCAF1 requires interaction with the NOT complex for its activity, since tethered λN-TbCAF1-myc might still interact with the NOT complex. To reduce the interaction, we depleted TbCNOT10 by RNAi. Now, λN-CAF1-myc alone should be tethered. Nevertheless, the GFP-boxB mRNA reporter was degraded as actively as before (Figure 7, lanes 3–6 and 9–12). This result suggests that TbCAF1 does not depend on NOT complex association in order to degrade a tethered substrate. In other words, TbCAF1 alone is indeed active in vivo, but it depends on the presence of TbCNOT10—and, by extension, perhaps the rest of the NOT complex—to be recruited to its substrate.

**HsCNOT10 is probably not required for association of HsCAF1 with the NOT complex**

Knowing the importance of TbCNOT10 in mRNA degradation, we were interested whether this function was conserved in mammalian cells. Therefore, we first confirmed the interaction of HsCAF1a and HsCNOT10 in HEK 293 cells by co-IP (Figure 8A). Using myc-strep-tagged HsCAF1a or HsCAF1b, we were able to pull down endogenous HsCNOT10 and confirm the interaction previously shown by TAP (38). To determine whether the association of HsCAF1a with core components of the human NOT complex is HsCNOT10-dependent, we knocked down HsCNOT10 by siRNA
and analysed the migration of HsCAF1a in a glycerol gradient. As controls we used a scrambled siRNA and a siRNA against HsNOT1. Figure 8B shows that HsCNOT10 depletion did not influence the migration of HsCAF1a, whereas knock-down of HsNOT1 led to a shift of HsCAF1a towards lighter fractions. The knock-down efficiency of HsCNOT10 and HsNOT1 was verified by western blot analysis (Figure 8C). We also attempted to
Figure 8. *HsCNOT10* is not required for association of CAF1 with the human NOT complex. (A) Myc-strep-tagged *HsCAF1a* or *HsCAF1b* were purified from HEK293 cells, and association of *HsCNOT10* was assessed by western blot analysis. Cells expressing the tag alone served as the control. (B) Control, human CNOT10 and NOT1 RNAi extracts were run on 10–30% glycerol gradients and 15 fractions were collected. Western blots of the gradient fractions were analysed for the migration of *HsCAF1a*. The Ponceau-stained band is BSA, which was added to every fraction to serve as a control for equal precipitation efficiency. (C) *HsNOT1* and *HsCNOT10* depletion was confirmed by western blot analysis.

determine the effect of *HsCNOT10* depletion on Caf1b complex association, using a Caf1b-specific antibody. Results suggested that Caf1b also might have remained complex associated after *HsCNOT10* knock-down, but since Caf1b was extremely difficult to detect no firm conclusion could be drawn.

Notably, the knock-down of *HsCNOT1* reduced the amount of *HsCNOT10*, whereas the reverse was not the case. These results suggested that *HsCNOT10* is not required for association of *HsCAF1a* with *HsNOT1* in HEK293 cells.

**DISCUSSION**

In this study, we have shown that in the relatively simple trypanosome NOT complex, association of *TbCAF1* with *TbNOT1* is enhanced by the presence of *TbCNOT10*. Further, our results indicate that *in vivo*, this association is required for deadenylation of mRNAs by *TbCAF1*.

Our yeast two-hybrid experiments revealed various interactions between *TbCNOT10*, *TbCAF1*, *TbNOT5* and the N- and C-terminal parts of *TbNOT1* (Figure 4). The two-hybrid interactions of *TbCAF1*, *TbNOT5* and *TbCNOT10* with *TbNOT1* seem likely to be direct, as they parallel those seen in other species. The interaction between *TbCAF1* and *TbNOT1* most likely resembles that seen in opisthokonts (16,17). The relevant region of NOT1 is highly conserved across evolution and the most important CAF1-interacting residues of yeast and trypanosome NOT1s are identical. In CAF1, somewhat less conservation is evident in the interaction domain, but the overall hydrophobic character is conserved. In contrast, we cannot currently be sure that the apparent interaction between *TbCAF1* and *TbCNOT10* is direct, since we cannot rule out the possibility that they interact via yeast Not1p. Given the sequence conservation, *TbCAF1* might indeed interact with yeast Not1p. In contrast, *TbCNOT10* bears no resemblance to any yeast protein (including Caf130p) so interaction with Not1p would not be expected.

TAP of CNOT10, bearing an N-terminal protein-A-calmodulin-binding-peptide tag, did not result in co-purification of *TbCAF1*; exactly the same result was obtained for *HsCNOT10* bearing an N-terminal FLAG-HA tag (9). This was not caused by the buffer conditions since *TbCNOT10* was obtained after TAP of the complex using TAP-*TbCAF1*. It was probably not caused by the tag either: N-terminally TAP-tagged *TbCNOT10* was functional, since trypanosomes expressing only the TAP-tagged version were viable, and *TbCNOT10* that was N-terminally fused with either the V5 peptide or yeast-two hybrid components, was able to interact with N- or C-terminally tagged *TbCAF1*. The only explanation we can think of is that the interaction was somehow specifically competed by the binding of the tag to the resins.

In trypanosomes, *TbCNOT10* was required both for association of V5-*TbCAF1* with the complex, and for the presence of normal amounts of V5-*TbNOT1*. The easiest explanation of this result is that *TbCNOT10*, by interacting with both *TbCAF1* and *TbNOT1*, stabilizes *TbCAF1–TbNOT1* binding; and that *TbNOT1*, which is not associated with *TbCAF1*, is preferentially degraded.

Glycerol gradient analysis for both trypanosomes and HEK cells showed that about half of V5-*TbCAF1* or native *HsCAF1* was not associated with the NOT complex. The role of this ‘free’ fraction is not known. It may have no function at all, or it could have a role independent of deadenylation, for example in repressing translation, as shown for *Xenopus* oocytes (39). In addition, it could also act as a reserve stock for newly formed NOT
complexes. Since in trypanosomes, we saw a reduction in deadenylation and mRNA degradation in TbCNOT10-depleted cells, with concomitant loss of CAF1 association with the NOT complex, it seems likely that in vivo, free TbCAF1 is unable to digest PABP-coated poly(A) tails. In contrast, once TbCAF1 attaches to an mRNA, either via the complex or by tethering, digestion is possible (Figure 9). Similarly, in human cells, depletion of HsNOT1 or HsNOT2 destabilized the NOT complex and decreased deadenylation (14,19), and the interaction of HsCAF1a with HsNOT1 was required for Caf1-/Not-dependent degradation of an miRNA target (16). In budding yeast, disruption of the interaction between the deadenylases (Ccr4p and Caf1p) and Not1p, effected by expression of dominant-negative interaction-defective mutants, impaired growth and deadenylation (17).

In human cells, the NOT complex can be recruited to mRNAs through RNA-binding proteins (e.g. (13)). We expect the same to be true in trypanosomes, but so far there are no good candidate RNA-binding proteins that might fill this role. In our TAP of the trypanosome NOT complex, we did not identify any RNA-binding proteins, but this is not surprising. Eukaryotic NOT complexes probably interact with many different proteins, so that any one would be present at relatively low levels. Moreover, such interactions may be transient. Indeed, human TTP, which does interact with human NOT complex, was not detected in pull-downs with any human NOT complex component (9).

We do not know which NOT complex subunits are directly responsible for interactions with RNA or RNA-binding proteins. Potential candidates for recruitment of the complex to human mRNAs are HsCNOT3/5 and 9, both of which pulled down proteins with RNA-binding domains (9). In trypanosomes, recruitment of the NOT complex to different mRNAs through different subunits could at least partially explain why all of the subunits are required for cell growth. The role of CNOT10 in human cells remains to be elucidated, but one possibility is that both human and trypanosome CNOT10 play roles in the targeting of mRNAs for degradation.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online: Supplementary Tables 1 and 2 and Supplementary Figures 1–3.

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